Pathol. Oncol. Res. (2008) 14:449–455
DOI 10.1007/s12253-008-9047-7

ORIGINAL PAPER

Immunohistochemical Examination of Gastrin, Gastrin Precursors, and Gastrin/CCK-2 Receptor in Human Esophageal Squamous Cell Carcinomas

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Received: 23 March 2008 / Accepted: 1 April 2008 / Published online: 26 April 2008

Abstract A promoting effect of gastrin on stimulating Barrett's oesophagus proliferation has been demonstrated, but whether it plays a regulating role for esophageal squamous cell carcinoma (ESCC) to date has not been fully investigated. The aim of this study is to examine the expressions of gastrin, gastrin precursors and gastrin/CCK-2 receptor in ESCC. Tissue specimen sections from 38 patients with ESSC obtained from a high incidence area of north China were assessed using immunohistochemistry for amidated gastrin, gastrin precursors (progastrin and glycine-extended gastrin) and gastrin/CCK-2 receptors. Their clinical histopathological significance was also analyzed. Of 38 ESCC, the immunoreactivities of gastrin, glycine-extended gastrin and progastrin were observed in 13.2% (5/38), 7.9% (3/38) and 23.68% (9/38) cases. The expression of progastrin was obviously higher than other gastrins, though not significantly ($P>0.05$). In positive cases for gastrin or glycine-extended gastrin, the scores of positive tumor cell numbers were at a lower density (<10/abundant-distributed field). However, the scores of progastrin positive tumor cell density in five of nine positive cases were over 10/abundant-distributed field. The immunoreactivity of gastrin/CCK-2 receptor was also observed in 15.8% (6/38) ESCC cases. There was not significant correlation regarding immunohistochemical results with known histomorphological parameters i.e. gender, tumor location and TNM stages. Based on our current results, ESCC tumor cells could be a possible cellular source of gastrin precursors, which has been postulated to play a role in regulating the growth in some human tumor cells.

Keywords Esophagus · Squamous cell carcinoma · Gastrin · Immunohistochemistry

Abbreviations

ESCC	esophageal squamous cell carcinoma
pro-G	progastrin
G-gly	glycine-extended gastrin
IHC	immunohistochemistry

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Introduction

Esophageal cancer is one of the most common malignancy worldwide with a very poor prognosis [1]. Many risk factors i.e. social status, nutrition, genetic, infectious, alcohol consuming and smoking have been related to the development of this malignance [2]. However, the exact etiology of esophageal cancers is still kept in unclear [2].

Most current clinical therapies except surgical resection offers a significant improvement of survival and the patient's prognosis is heavily dependent on the early diagnosis. To improve clinical treatment, attempts to better understand the controlling of tumor cell growth have been made at the mean time; results showed that the growth of esophageal cancers is dependent on the stimulation of growth factors i.e. cyclooxygenase-2 [3], insulin-like growth factor-I [4], epidermal growth factor [5], gastrin-releasing peptide [6] and fascin [7].

Gastrin, a family of peptides released from antraduodenal G cells, is initially synthesized in the stomach as progastrin (pro-G), which is then processed to glycine-extended gastrin (G-gly) and finally to amidated gastrin (G-17 and G-34) [8]. Gastrin has been demonstrated as the most important trophic factor for gastrointestinal mucosa as well as a promoter for growth of human cancers including gastric cancer [9, 10], colon cancer [11, 12], pancreatic cancer [13–15], liver cancer [16], lung cancer [17] and renal cancer [18] etc. The promotion effect of gastrin on tumor cell growth can be reached by either an autocrine or paracrine manners [19]. Indeed, the coexpression of gastrin and gastrin/CCK-2 receptor has been demonstrated in many cancers [12, 15, 20, 21], this suggests that gastrin produced by tumor cells may act as a growth factor that interacts with their specific membrane receptors on the tumor cell surface to induce proliferation or prolongation of their survival [15, 19, 22]. The effect of gastrin on tumor proliferation was initially considered to be mediated by its final physiological form—amidated gastrin, however, recent studies have indicated that incompletely processed gastrins (pro-G and G-gly) are biologically active and might play a role in both the growth and differentiation of the gastrointestinal mucosa [14, 23–29] and promote the growth of gastrointestinal cancers [8, 9, 14, 23–28], the incompletely processed gastrins are also possibly released by tumor cells selves.

In animals, amidated gastrin was previously shown to stimulate the proliferation of esophageal epithelium in rats by either omeprazole (a potent gastric acid inhibitor) induced hypergastrinemia or infused gastrin-17 [30, 31]. Karaki et al. [32] reported that hypergastrinemia in rats enhances chemical carcinogen induced esophageal carcinoma. In human, the important role of long-term hypergastrinemia on increasing risk of esophageal cancer has been reported to be mediated by stimulating the growth of Barrett's esophageal epithelium that were via several mechanisms including the activation of the gastrin/CCK2 receptor [30, 33] and/or reduction of apoptosis [21]. In esophageal adenocarcinoma, the gastrin/CCK2 receptor chromosome was lost in most cases and became hardly to detected, thus, the promoting effect of gastrin on Barrett's esophageal epithelium could be via a pathway of induction of cyclooxygenase-2 [34]. Histologically, esophageal cancers can be classified into two main histological types: esophageal squamous cell carcinomas (ESCC) that presents as great majority in some Asian countries such as China, and adenocarcinoma that is more frequently seen in western countries and showed in a dramatically increasing rate recently [1]. Until now, the role of incompletely processed gastrins in ESCC was largely ignored; whether gastrin and its precursors play a role in regulating growth of ESCC remains unclear.

Linzhou (formerly Linxian), a county in Henan Province, located in north–central china, has one of the highest rates of esophageal cancer in the world with age standardized incidence rates for both sexes exceed 100/100 000 per year and annual age-adjusted mortality rates of up to 169 per 10^5 [35]. The dominate histological type of esophageal cancers in Linzhou is ESCC [36], provides a perfect opportunity to evaluate ESCC and many studies have been performed in this county. Therefore, the aim of this study is to examine the expression of gastrin, gastrin precursors (pro-G and G-gly) and the gastrin/CCK-2 receptor in ESCC tissues obtained from this high incidence area. Their expression in ESCC tumor cells indicates that tumor cells could be an important cellular source of gastrin precursors.

Patients and Methods

ESCC Samples

A total of 38 cases of advanced ESCC were randomly retrieved from the files of Department of Pathology of the People's Hospital of Linzhou for this study. The average age at treatment was 57 years (ranging from 32 to 73 years). The male female ratio was 23/15. Among 38 cases of ESCC, 12 cases with the corresponding transitional tissues. Of 23 male patients, 87% (20/23) were with long-term tobacco consuming and no any females had tobacco consuming record. Both males and females were without long-term alcohol consuming history. No patients received radiotherapy and/or chemotherapy preoperative. Curative surgery was performed in all 38 patients. Resected specimens from ESCC patients were longitudinally sliced, fixed in 10% formalin and embedded in paraffin. Representative sections were cut at 4 μm and stained with hematoxylin–eosin for routine histological diagnosis. Detailed basic information of ESCC histological findings and TNM stages [37] were summarized in Table 1. This study was approved by the local Medical Research Committee.

Immunohistochemistry

Sections for immunohistochemistry (IHC) were deparaffinized in xylene, rehydrated in graded ethanol, and incubated in

Table 1 Basic histological and clinical information of ESCC patients

Number	Gender	Alcohol	Tobacco	Location			Differentiation		TNM stage			
	M/F	M/F	M/F	Upper	Middle	Lower	Well	Moderate	I	II	III	IV
38	23/15	0/0	20/0	5[a]	25[b]	8	2	36	0	5	30	3

M/F Male/female
[a] Two cases mixed with middle part
[b] Two cases mixed with lower part

0.3% H_2O_2 solution in methanol for 15 min to block endogenous peroxidases. Antigen retrieval was achieved of boiling sections for 15 min in 0.01 M citrate buffer, pH 6.0. Nonspecific binding was blocked by incubating sections in phosphate buffered saline (PBS) containing 4% normal bovine serum and 0.25% Triton-X 100. The slides were rinsed three times with PBS with 0.25% Triton-X 100 (PBS-T) for 5 min and incubated over night 4°C with an anti-human gastrin (1:600; DAKO, Carpinteria, CA, USA), anti-human gastrin/CCK2 receptor (1:100; Abcam, Cambridge, UK), anti-glycine extended gastrin and anti-progastrin (both at 1:400) antibodies that have been described elsewhere [13, 38]. After which the slides were again washed with PBS-T for 10 min and detection was performed with a commercial LSAB-2 system-horseradish peroxidase kits (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions and our published method [39]. 3-Amino-9-ethylcarbazole (Vector Laboratories, Burlingame, CA, USA) was used as chromogen and slides were counterstained with Mayer's hematoxylin. The negative control slides for IHCs were performed routinely: (1) primary antibodies were substituted with the isotype-matched control antibodies; (2) secondary antibody was substituted with PBS-T. The sections from antral mucosa were used as positive controls for IHCs of gastrin, pro-G and G-gly; section from oxyntic mucosa with parietal cells was used as positive control for IHC of gastrin/CCK2 receptor.

Morphometric Evaluation

All the stained slides were examined under light microscopy and only the slides with gastrins and gastrin/CCK2 receptor immunoreactivities (IRs) in tumor cell were identified as positive. The numbers of positive tumor cells in five independent high-power fields (×400) with abundant distribution were counted and scored as follows: (−), no immunoreactive cells; (+), one to five cells; (++), five to ten cells; (+++), less than ten cells but <1% of total cell mass; (++++), >1% [40].

Statistical Analysis

The positive rate of ESCC for gastrins and gastrin/CCK2 receptor was counted and expressed as percentage and the variability of positive rates was evaluated with Chi-square test.

Results

In 12 non-tumor corresponding squamous mucosa, IRs of gastrin, gastrin precursors of pro-G and G-gly and gastrin/CCK2 receptor were not found.

In ESCC tissues, the tumor cells that expressed gastrin, pro-G and G-gly were mostly sporadically (Fig. 1c,d) and occasionally occurred as micronodules (Fig. 1a). The positive rates of gastrin, pro-G, Gly-G and gastrin/CCK2 receptor were varied. Notably, the positive rate of pro-G IR in tumor cells was higher than others, though the difference was not significantly (Table 2; $P>0.05$; pro-G positive tumor cells were observed in 23.68% (9/38), G-gly in 7.9% (3/38) and gastrin in 13.2% (5/38) cases of ESCC). In sections, the pro-G positive tumor cells were more frequently found than gastrin and G-gly positive tumor cells, the score for pro-G positive tumor cell number was 3 (+++) in ~50% positive cases (Table 3 and Fig. 1c), while the scores for G-gly and gastrin positive tumor cell numbers were mostly less than 2 (++; detailed information shown in Table 3). It was interesting to note that the most pro-G expressing cases (6/9) were not positive for gastrin IR, but all the positive cases of G-gly were also positive for pro-G antibody (see Table 3).

The correlations of scores for pro-G positive tumor cells with clinical histological parameters of ESCC were further analyzed. It showed that the positive rates for pro-G were not significant different with respect to gender ($P>0.05$, Chi-square test; Fig. 2), location ($P>0.05$, Chi-square test; Fig. 2) and TNM stage ($P>0.05$, Chi-square test; Fig. 2). In addition, there were also not positive associations were found between G-gly positive or gastrin positive tumor cell number scores and clinical histological parameters of ESCC (data not shown). Since most of cases included in this study were moderately differentiated ESCC, it was hard to make any statements about the correlation between differentiation and positive rates of gastrins.

Moreover, some tumor cells were positive for gastrin/CCK2 receptor antibodies (Table 3) in 15.8% (6/38) of

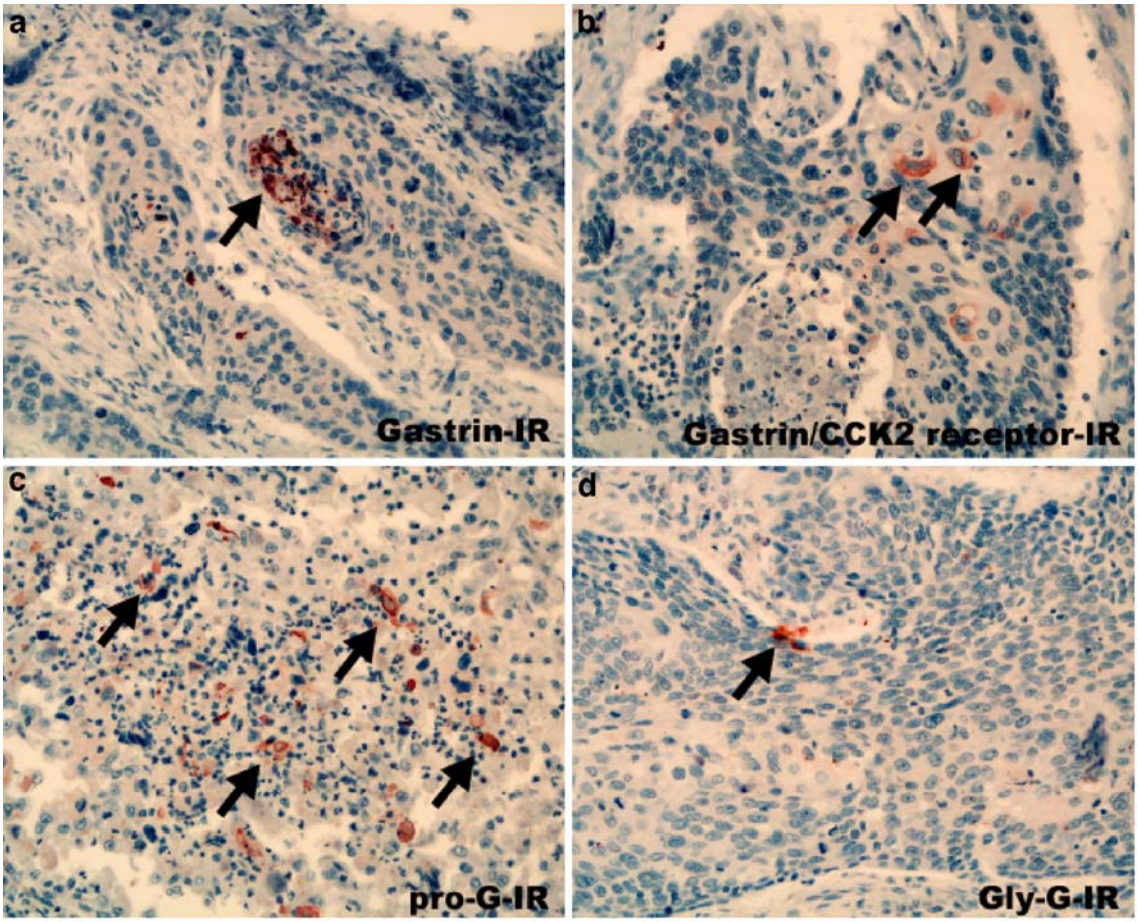

Fig. 1 Immunohistochemical examination of gastrins and gastrin/CCK2 receptor in ESCC tissues. Immunoreactivities (*IR*s) of **a** gastrin, **b** gastrin/CCK2 receptor, **c** progastrin (*pro-G*) and **d** glycine-extended gastrin (*G-gly*) were detected in tumor cells of ESCC. However, tumor cells with *pro-G IR* were more frequently observed. (IHC, counter-stained with hematoxylin; magnification ×200)

cases of ESCC. Halve of gastrin/CCK-2 receptor positive cases were also positive for both pro-G and G-gly antibodies, and only two of them were positive for gastrin in the same case (see details in Table 2). There were also not positive associations were found between gastrin/CCK-2 receptor positive tumor cell number scores and clinical histological parameters of ESCC (data not shown).

Table 2 Expression of gastrins and gastrin/CCK2 receptor in ESCC (*N*=38)

Antibodies	Cases with tumour cell staining (of total cases)
Gastrin	13.20%
Progastrin	23.68%
Glycine extended gastrin	7.90%
Gastrin/CCK2 receptor	15.80%
P value (Chi-square test)	>0.05

Discussion

Our current results demonstrated the low density amidated gastrin-, Gly-G- and gastrin/CCK2 receptor-positive tumor cells, but a high density pro-G positive tumor cells in ESCC. To our best knowledge, this is the first time to show the immunoreactivities of incompletely processed gastrins (precursors) in ESCC tumor cells.

Increased incompletely processed gastrins (precursors) expression either in local tissue section and serum have been reported in human cancers [14, 23, 41, 42]. Caplin et al. [13, 16] have demonstrated a high expression of gastrin precursors in pancreatic cancer and hepatocellular cancer. Henwood et al. [24] reported an early increased expression of gastrin precursors along precancerous changes of gastric cancer. In our current study, a high positive rate (23.68%) for pro-G in ESCC sections was found, that was higher than other gastrin products. Given the finding of potential roles of gastrin precursors in stimulating human cancer growth in vitro [25, 43, 44] and animal cancer models in vivo [26, 28,

Table 3 Clinical histopathology of ESCC positive for gastrins, gastrin/CCK2 receptor IRs

ID	Age	Gender	Differentiation	TNM stage	Location	Gastrin	pro-G	G-gly	Gastrin/CCK2-R
1	58	M	Mod	II	L		+	+	+
2	50	M	Mod	III	L		++++	+	+
3	67	F	Mod	III	Upper		++		
4	65	M	Mod	III	L	++			+
5	53	F	Mod	III	L	+			
6	66	M	Mod	III	Upper	+	+	+	+++
7	70	M	Mod	III	Upper + M		+		
8	32	F	Mod	III	Mid + L		+++		
9	56	M	Mod	III	Mid + L	+			
10	68	M	Mod	III	Mid		+		
11	57	M	Mod	IV	Mid		+++		
12	59	F	Mod	III	Mid	+++			+
13	43	M	W	III	L				++++
14	51	F	W	III	Upper		++++		

M Male, *F* female, *M* moderately differentiated, *W* well differentiated, *All* all layers, *Upper* upper part of oesophagus, *Mid* middle part of oesophagus, *L* lower part of oesophagus, *pro-G* progastrin, *G-gly* glycine-extended gastrin, *Gastrin/CCK2-R* gastrin/CCK2 receptor

39, 45], we therefore postulate that high positive rate of pro-G in ESCC might play a role in regulating ESCC tumor cell growth.

It is well known that the effect of amidated gastrin on stimulating gastrointestinal cancer cell growth was mediated by gastrin/CCK2 receptors [15, 17, 18], a gastrin–gastrin/CCK2 receptor loop was found in some human cancers [15, 16, 20, 24] and targeting of gastrin/CCK2 receptor by peptide or antibody exhibited a therapeutic potential in colonic, pancreatic and hepatocellular carcinoma cell lines in vitro [46]. In animals, gastrin has been found to stimulate normal esophageal epithelium proliferation [31, 47, 48] and promote carcinogenesis [32]. In Barett's esophagus, gastrin induces proliferation [33], metaplasia [33], and reduced apoptosis [21], which were mediated by upregulated gastrin/CCK2 receptors [21, 33]. In addition, gastrin can also enhance proliferation of esophageal adenocarcinoma [30], the expression of gastrin/CCK2 receptor in esophageal adenocarcinoma, however, was hardly detected. Thus, concerning the pathway of gastrins in stimulating tumor cell growth is still a uncertain issue. Some studies suggest that they act directly on their own receptor expressed on tumor cell surface, and other studies postulate a indirect mechanism that is probably via a indirect pathway such as upregulated cyclooxygenase-2 expression [34]. In this study, gastrin/CCK-2 receptor positive tumor cells were detected in a low rate (15.8%) and low density in ESCC. This finding indicated that the main action pathway of progastrin in ESSC is probably not via a traditional gastrin/CCK2 receptors. Specific receptors for gastrin precursors have been postulated to exist in other gastrointestinal cancer cell lines [49]. Therefore, it will be important to identify the specific receptor for progastrin in tumor cells in future studies.

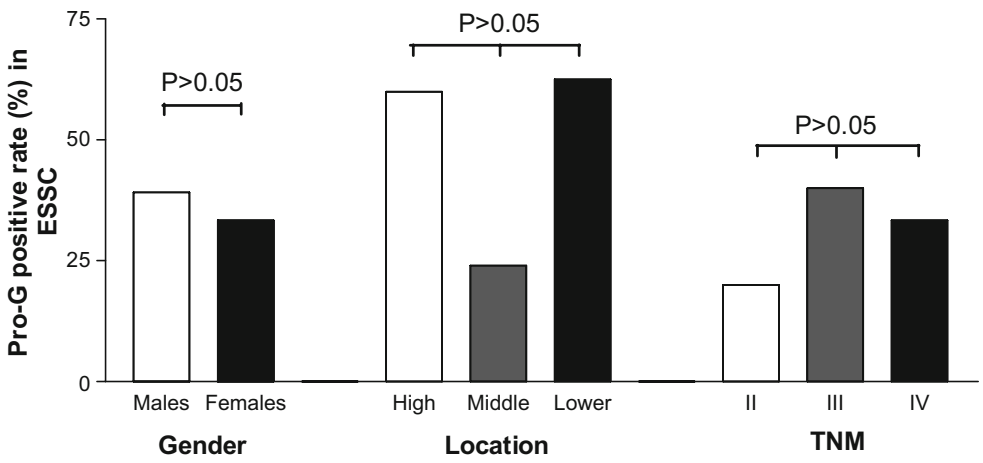

Fig. 2 Graphic analysis of the association between pro-G positive rates and clinical histological parameters in ESCC. The positive rates of pro-G in ESCC were not statistically different with respect to gender, location and TNM stages (All $P>0.05$, Chi-square test)

In summary, based on our current findings, ESCC tumor cells could be a possible cellular source for pro-G.

Acknowledgments This study was partially supported by Henan Provincial Medical Healthy Committee Foundation. We express our sincere gratitude to our colleagues at Department of Pathology of the People's Hospital of Linzhou for supplying biopsies.

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